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AUTHOR(S):

Sato, Naoe; Yokoyama, Chikako; Inukai, Miki; Miyashita, Saeko; Nagase, Keito; Nakano, Takafumi; Iuchi, Katsuya; Hisatomi, Hisashi

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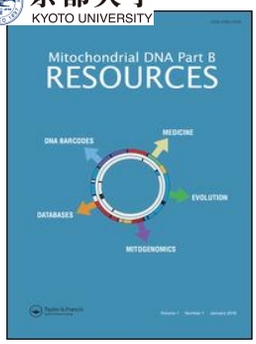

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Analysis of genetic variation in mitochondrial cytochrome c oxidase subunit 1 between *Haemadipsa japonica* in Japan and land leeches worldwide

Naoe Sato[a,b], Chikako Yokoyama[c], Miki Inukai[a], Saeko Miyashita[a], Keito Nagase[a], Takafumi Nakano[d], Katsuya Iuchi[a] and Hisashi Hisatomi[a]

[a]Department of Materials and Life Science, Seikei University, Tokyo, Japan; [b]Seikei Junior and Senior High School, Tokyo, Japan; [c]Department of Biochemical Engineering, Graduate School of Science and Engineering, Yamagata University, Yamagata, Japan; [d]Division of Biological Science, Department of Zoology, Graduate School of Science, Kyoto University, Kyoto, Japan

ABSTRACT

Haemadipsa japonica is the most common land leech species found in Japan. It has been considered to possess genetic variation that limits its habitat range. In the present study, to characterize variation in the mitochondrial cytochrome c oxidase subunit 1 (cox1) gene of *H. japonica* specimens from various locations in Japan, we examined nucleotide sequences of the mitochondrial cox1 gene. We performed PCR of mitochondrial cox1 using 10 *H. japonica* specimens and compared the result with those of land leeches from around the world using a maximum likelihood (ML) tree. ML tree of *H. japonica* in Japan showed significant differences between cox1 sequences of specimens from Yakushima and other regions of Japan. ML tree of land leeches from around the world revealed that *H. japonica* had the closest relationship with *H. picta* from Borneo.

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Mitochondrial DNA; Mitochondrial cox1; *Haemadipsa japonica*; the maximum likelihood method (ML) tree

Haemadipsa japonica (*H. japonica*) is a common hematophagous land leech found in Japan. *H. japonica* has evolved to be locally distributed. *H. japonica* specimens found in various regions of Japan lack morphological differences and cannot thus be classified based on morphological features. However, they are thought to possess genetic variation that limits their habitat range. To assess phylogeographic patterns in *H. japonica* located at sites across Japan, we examined nucleotide sequences of the cox1 gene of mitochondrial DNA (mtDNA).

To determine the species identity and evaluate the genetic relationships among *H. japonica* specimens throughout Japan, cox1 gene sequences were amplified from 10 *H. japonica* specimens. Specimens were collected from along bushes and the forest floor in Yakushima (Kagoshima) (30.452222N, 130.485277E), Mie (35.196944N, 136.543611E), Shiga (35.344444N, 135.922777E), Kanagawa (35.512777N, 139.247499E), Shizuoka (35.586946N, 138.314166E), Gunma (36.704722N, 138.986388E), Kimitsu (Chiba) (35.204445N, 140.1405580E), Gifu (35.527499N, 136.639722E), Kamogawa (Chiba) (35.134444N, 140.150277E), and Akita (39.796668N, 140.235001E).

mtDNA was extracted using phenol/chloroform method. Two primers (5′-ATT AAG AGG AAT ACC GCC ATT AAC TGG-3′ for forward primer and 5′-AAT AGC AGG TGC AAT TGT TCA TAA AGT TTC-3′ for reverse primer) for amplification and five primer sets for sequencing of mtDNA were designed. After long PCR using Pfu-x DNA polymerase (Greiner Bio-One, Frickenhausen, Germany), PCR products (2064 bp) were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Complete cox1 sequence of *H. japonica* at Kanagawa was 1569 bp long. This sequence has been submitted to GenBank (accession no. LC424190) as a representative.

Multiple comparisons were performed using MEGA software version 7 to evaluate levels of similarity and differences between nucleotide sequences (Hall 2013). A phylogenetic tree obtained by combining our data with previous data (604 bp) (Lai et al. 2011; Won et al. 2014) is shown in Figure 1(A). Maximum likelihood (ML) estimation determines values for parameters of a statistical model such that the parameter values maximize the likelihood that the process described by the model was produced by the observed data. Using the 604 bp cox1 sequences, ML tree showed that *H. japonica* in Japan belonged to a distinct group from other *Haemadipsa*. ML tree of land leeches from among the world revealed that *H. japonica* had the closest relationship with *H. picta* from Borneo.

Moreover, ML tree showed a significant difference between complete cox1 sequences (1569 bp) of *H. japonica* at Yakushima and other regions of Japan (Figure 1B). Furthermore, our findings suggest that *H. japonica* inhabiting Honshu Island can be separated into four major haplotypes.

CONTACT Chikako Yokoyama 4koyama@yz.yamagata-u.ac.jp Department of Biochemical Engineering, Graduate School of Science and Engineering, Yamagata University, 4-3-16 Jonan, Yonezawa, Yamagata 992-8510, Japan

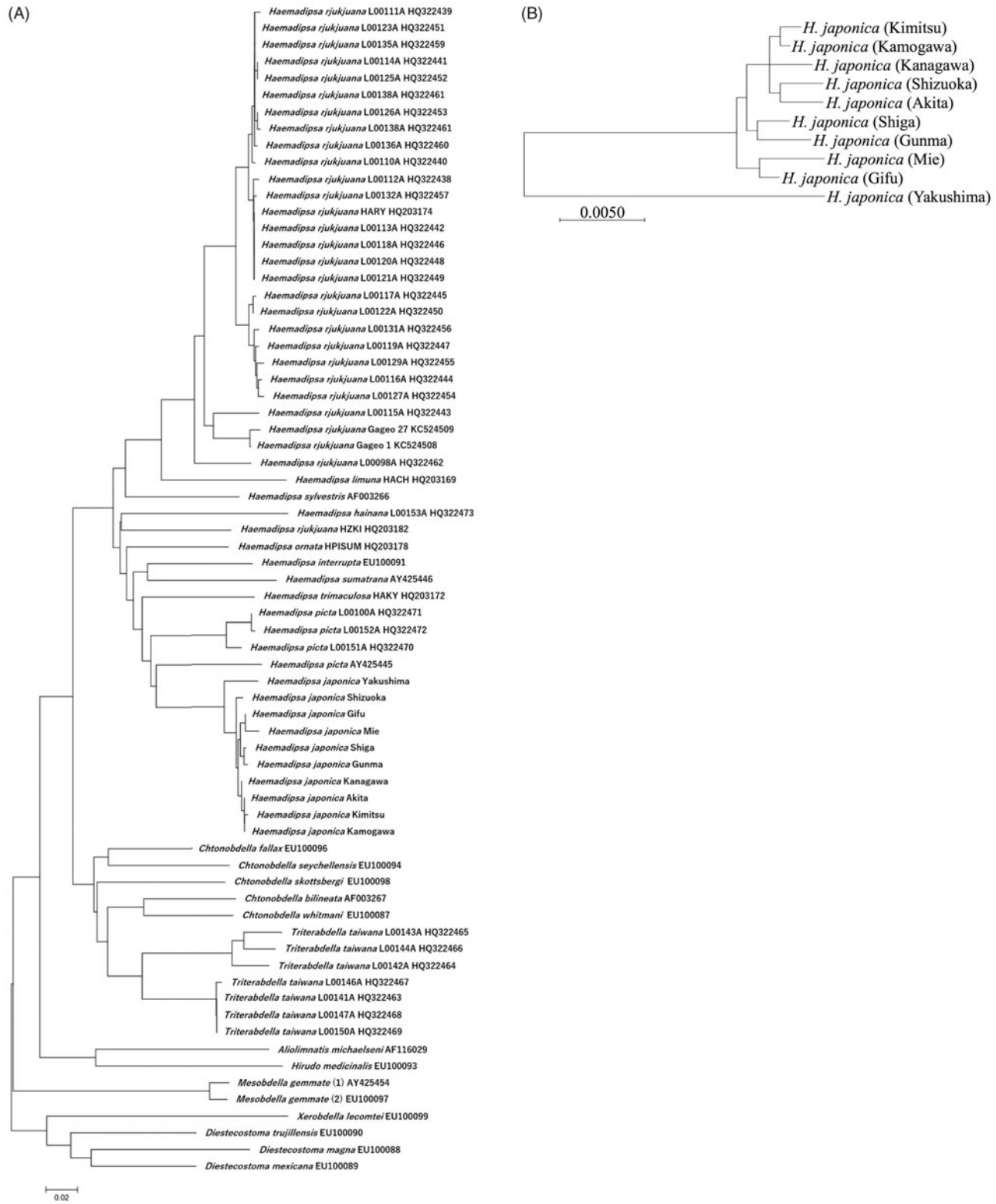

Figure 1. Maximum likelihood (ML) tree based on mitochondrial cox1 sequences. (A) ML tree based on 604 bp cox1 sequences of land leech specimens from around the world and 10 *H. japonica* specimens from Japan. (B) ML tree based on 1569 bp cox1 sequences of 10 *H. japonica* specimens from Japan. The bootstrap value based on 1000 replicates is shown on each node.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Takafumi Nakano http://orcid.org/0000-0001-6107-2188

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